

MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

FIELD OF THE INVENTION

The present invention is generally directed to the detection of genetic markers in a genomic system. The present invention is more specifically directed to the simultaneous amplification of multiple distinct polymorphic genetic loci 5 using the polymerase chain reaction or other amplification systems to determine in one reaction the alleles of each locus contained within the multiplex system.

CITED REFERENCES

10 A full bibliographic citation of the references cited in this application can be found in the section preceding the claims. /

DESCRIPTION OF THE PRIOR ART

15 In recent years, the discovery and development of polymorphic short tandem repeats (STRs) as genetic markers has stimulated progress in the development of linkage maps, the identification and characterization of diseased genes, and the simplification and precision of DNA typing.

20 Many loci, at least in the human genome, contain a polymorphic STR region. STR loci consist of short, repetitive sequence elements of 3 to 7 base pairs in length. It is estimated that there are 2,000,000 expected trimeric and tetrmeric STRs present as frequently as once every 15 25 kilobases (kb) in the human genome (Edwards et al. 1991; Beckmann and Weber 1992). Nearly half of the STR loci studied by Edwards et al. (1991) are polymorphic, which provides a rich source of genetic markers. Variation in the number of repeat units at a particular locus is responsible for the 30 observed polymorphism reminiscent of VNTR loci (Nakamura et al. 1987) and minisatellite loci (Jeffreys et al. 1985), which contain longer repeat units, and microsatellite or dinucleotide repeat loci (Litt and Luty 1989, Tautz 1989,

Weber and May 1989, Beckmann and Weber 1992).

5 Polymorphic STR loci are extremely useful markers for human identification, paternity testing and genetic mapping. STR loci may be amplified via the polymerase chain reaction (PCR) by employing specific primer sequences identified in the regions flanking the tandem repeat.

10 Alleles of these loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another following electrophoretic separation by any suitable detection method including radioactivity, fluorescence, silver stain, and color.

15 To minimize labor, materials and analysis time, it is desirable to analyze multiple loci and/or more samples simultaneously. One approach for reaching this goal involves amplification of multiple loci simultaneously in a single reaction. Such "multiplex" amplifications have been described extensively in the literature. Multiplex amplification sets have been extensively developed for analysis of genes related 20 to human genetic diseases such as Duchenne Muscular Dystrophy (Chamberlain et al. 1988, Chamberlain et al. 1989, Beggs et al. 1990, Clemens et al. 1991, Schwartz et al. 1992, Covone et al. 1992), Lesch-Nyhan Syndrome (Gibbs et al. 1990), Cystic Fibrosis (Estivill et al. 1991, Fortina et al. 1992, Ferrie et 25 al. 1992, Morral and Estivill, 1992), and Retinoblastoma (Lohmann et al. 1992). Multiplex amplification of polymorphic microsatellite markers (Clemens et al. 1991, Schwartz et al. 1992, Huang et al. 1992) and even STR markers (Edwards et al. 1992, Kimpton et al. 1993, Hammond et al. 1994) have been 30 described.

35 These amplified products are generally separated by one of several methods of electrophoresis known to those skilled in the art. Several well-known methods of detection of the amplified products have also been described. While ethidium bromide staining of amplified fragments is employed

in some cases, in others it is preferred to use methods which label only one of the two strands of the amplified material. Examples of this include radioactive or fluorescent labeling of one of the two primers prior to the amplification of a 5 locus. One of the more sophisticated approaches to detection is the use of different fluorescent labels to allow detection of amplified materials representing different loci, but existing in the same space following electrophoresis. The products of the different loci are differentiated with the use 10 of filters, which allow visualization of one fluorescent label at a time.

Reference is made to International Publications WO 93/18177 and WO 93/18178 to Fortina et al., which are directed 15 to methods and kits for diagnosing diseases such as Cystic Fibrosis and  $\beta$ -thalassemia, respectively, using an allele-specific multiplex polymerase chain reaction system. According to Fortina et al., multiplex PCR has also been used 20 for simultaneous amplification of multiple target sequences, permitting mutant allele scanning using two lanes of an agarose gel.

Ballabio et al. (1991), disclose a single tube multiplex allele specific PCR test using two different dye-tagged fluorescent primers for detection of the  $\Delta$ F508 cystic fibrosis mutation.

25 While there are multiplex amplification procedures for specific loci, the use of multiplex amplification procedures is greatly desired for the detection of alleles in other types of loci such as specific STR loci.

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#### SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for the simultaneous amplification of multiple distinct polymorphic STR loci using PCR or other amplification systems to determine, in one reaction, the 35 alleles of each locus contained within the multiplex. These

combinations of specific loci into multiplexes have not been heretofore shown.

It is also an object of the present invention to provide a method and a kit specific for multiplex 5 amplifications comprising specified loci.

These and other objects are addressed by the present invention which is directed to a method of simultaneously analyzing or determining the alleles present at each individual locus of each multiplex. This method comprises the 10 steps of (1) obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least two loci which can be amplified together; (2) amplifying the STR sequences in the DNA sample; and (3) detecting the amplified materials in a fashion which reveals the polymorphic nature of the systems 15 employed.

The present invention is also directed to a method of simultaneously analyzing multiple STR sequences wherein at least one of the loci is selected from the group consisting of: HUMCSF1PO, HUMTPOX, HUMVWFA31, HUMFESFPS, HUMBFXIII 20 (F13B), HUMLIPOL, HSAC04 (ACTBP2), HUMCYP19, HUMPLA2A1, HUMAPOA2, HUMCD4, HUMF13A01 and HUMMYOPK (Myotonic).

Specifically, the present invention is directed to a method of simultaneously analyzing multiple STR sequences in the following groups of loci: HUMTH01 and HUMCSF1PO; HUMTH01 25 and HUMCD4; HUMTH01 and HUMTPOX; HUMF13A01 and HUMFABP; HUMF13A01 and HUMMYOPK (Myotonic); HUMF13A01 and HUMBFXIII (F13B); HUMBFXIII (F13B) and HUMFESFPS; HUMBFXIII (F13B) and HUMLIPOL; HUMHPRTB and HUMFESFPS; HSAC04 (ACTBP2) and HUMCYP19; HUMCSF1PO, HUMTPOX and HUMTH01; HUMHPRTB, HUMFESFPS 30 and HUMVWFA31; HSAC04 (ACTBP2), HUMCYP19 and HUMPLA2A1; HSAC04 (ACTBP2) and HUMFABP; HUMAPOA2, HUMCYP19 and HUMPLA2A1; HUMCD4, HUMCSF1PO and HUMTH01; HUMCYP19, HUMFABP and HUMPLA2A1; HUMCYP19, HUMHPRTB and HUMPLA2A1; HUMF13A01, HUMFABP and HUMCD4; HUMHPRTB, HUMFESFPS and HUMLIPOL; 35 HUMF13A01, HUMFABP and HUMCD4; HUMHPRTB, HUMBFXIII (F13B) and

HUMPLA2A1; HUMHPRTB, HUMBFXIII (F13B) and HUMTPOX; HUMHPRTB, HUMBFXIII (F13B) and HUMFESFPS; HUMCSF1PO, HUMTPOX and HUMCD4; HUMHPRTB, HUMFESFPS and HUMMYOPK (Myotonic); HUMCSF1PO, HUMTH01 and HUMCD4; HUMCSF1PO, HUMTH01 and HUMVWFA31; 5 HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL; HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31; HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B) and HUMLIPOL; HUMCSF1PO, HUMTPOX, HUMTH01 and HUMCD4; and HUMCSF1PO, HUMTH01, HUMTPOX and HUMCD4.

The present invention provides a high throughput 10 method for the detection and analysis of polymorphic genetic markers using specific combinations of loci and specified conditions. By selection of the appropriate detection method, the process can be used in laboratories which have only a power supply and a standard apparatus for polyacrylamide gel 15 electrophoresis or those which have the latest in equipment for fluorescent gel scanning, e.g., FluorImager™-575 (Molecular Dynamics, Sunnyvale, CA). Thus, the process of the present invention is adaptable for a variety of uses and laboratories.

The approach as specified in the present invention 20 produces a savings in time, labor and materials in the analysis of loci contained within the multiplexes. The process of the present invention includes all the requisite primers, allowing between two and four or more loci to be amplified together in one amplification tube instead of 25 amplifying each locus independently.

The present invention has specific use in the field 30 of forensic analysis, paternity determination, monitoring of bone marrow transplantation, linkage mapping, and detection of genetic diseases and cancers.

These and other aspects of the present invention will become evident upon reference to the following detailed description of the invention and the attached drawings and photographs.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph illustrating the simultaneous amplification of three loci: HUMCSF1PO, HUMTPOX and HUMTH01, with the amplified products of each locus shown 5 migrating next to the corresponding allelic ladder for ease of interpretation in Example 1.

Figure 2 is a computer image showing the fluorescent detection of multiplex amplification of the loci HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31 as detected with a 10 FluorImager™-575 (Molecular Dynamics, Sunnyvale, CA) in Example 2.

Figure 3 is a photograph showing the silver stain detection of the multiplex amplification in Example 3.

Figure 4 is a computer image showing the fluorescent 15 detection of multiplex amplification in Example 4.

Figure 5 is a photograph showing the silver stain detection of the multiplex amplification in Example 5.

Figure 6 is a photograph showing the silver stain detection of the multiplex amplification in Example 6.

Figure 7 is a photograph showing the silver stain 20 detection of the multiplex amplification in Example 7.

Figure 8 is a photograph showing the silver stain detection of the multiplex amplification in Example 8.

Figure 9 is a photograph showing the silver stain 25 detection of the multiplex amplification in Example 9.

Figure 10 is a photograph showing the silver stain detection of the multiplex amplification in Example 10.

Figure 11 is a photograph showing the silver stain detection of the multiplex amplification in Example 11.

Figure 12 is a photograph showing the silver stain 30 detection of the multiplex amplification in Example 12.

Figure 13 is a photograph showing the silver stain detection of the multiplex amplification in Example 13.

Figure 14 is a photograph showing the silver stain 35 detection of the multiplex amplification in Example 14.

Figure 15 is a photograph showing the silver stain detection of the multiplex amplification in Example 15.

Figure 16 is a photograph showing the silver stain detection of the multiplex amplification in Example 16.

5 Figure 17 is a photograph showing the silver stain detection of the multiplex amplification in Example 17.

Figure 18 is a photograph showing the silver stain detection of the multiplex amplification in Example 18.

10 Figure 19 is a photograph showing the silver stain detection of the multiplex amplification in Example 19.

Figure 20 is a photograph showing the silver stain detection of the multiplex amplification in Example 20.

Figure 21 is a photograph showing the silver stain detection of the multiplex amplification in Example 21.

15 Figure 22 is a photograph showing the silver stain detection of the multiplex amplification in Example 22.

Figure 23 is a photograph showing the silver stain detection of the multiplex amplification in Example 23.

20 Figure 25 is a photograph showing the silver stain detection of the multiplex amplification in Example 25.

Figure 26 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 26.

25 Figure 27 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 27.

Figure 28 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 28.

30 Figure 29 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 29.

Figure 30 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 30.

Figure 31 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 31.

5 Figure 32 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 32.

DETAILED DESCRIPTION OF THE INVENTION

10 The following definitions are intended to assist in providing a clear and consistent understanding of the scope and detail of the terms:

Allelic ladder: a standard size marker consisting of amplified alleles from the locus.

15 Allele: a genetic variation /associated with a segment of DNA, i.e., one of two or more alternate forms of a DNA sequence occupying the same locus.

20 Biochemical nomenclature: standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). Corresponding nucleotides are, for example, deoxyguanosine-5'-triphosphate (dGTP).

DNA polymorphism: the condition in which two or more different nucleotide sequences coexist in the same interbreeding population in a DNA sequence.

25 Locus (or genetic locus): a specific position on a chromosome. Alleles of a locus are located at identical sites on homologous chromosomes.

30 Locus-specific primer: a primer that specifically hybridizes with a portion of the stated locus or its complementary strand, at least for one allele of the locus, and does not hybridize efficiently with other DNA sequences under the conditions used in the amplification method.

35 Polymerase chain reaction (PCR): a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to amplify the number

of copies of a target DNA sequence by  $>10^6$  times. The polymerase chain reaction process for amplifying nucleic acid is covered by U. S. Patent Nos. 4,683,195 and 4,683,202, which are incorporated herein by reference for a description of the process.

5 Polymorphism information content (PIC): a measure of the amount of polymorphism present at a locus (Botstein et al., 1980). PIC values range from 0 to 1.0, with higher values indicating greater degrees of polymorphism. This  
10 measure generally displays smaller values than the other commonly used measure, i.e., heterozygosity. For markers that are highly informative (heterozygosities exceeding about 70%), the difference between heterozygosity and PIC is slight.

15 Primary reaction: initial reaction using the purified human genomic DNA as template for the PCR.

Primers: two single-stranded oligonucleotides or DNA fragments which hybridize with opposing strands of a locus such that the 3' termini of the primers are in closest proximity.

20 Primer pair: two primers including primer 1 that hybridizes to a single strand at one end of the DNA sequence to be amplified and primer 2 that hybridizes with the other end on the complementary strand of the DNA sequence to be amplified.

25 Primer site: the area of the target DNA to which a primer hybridizes.

Secondary reaction: reamplification with the same or different primer pair using a dilution of the primary reaction as template for the PCR.

30

#### Construction of the Multiplex System

Prior to constructing the multiplex system, an appropriate set of loci, primers, and amplification protocols must be selected such that amplification generates fragments 35 such that alleles of the various loci do not overlap in size

or, when such overlap occurs, fragments representing different loci are detectable by separate means. In addition, the selected loci must be compatible for use with a single amplification protocol. The specific combinations of loci 5 described herein are unique in this application. Combinations of loci may be rejected for either of these reasons, or because, in combination, one or more of the loci do not produce adequate product yield, or fragments which do not represent authentic alleles are produced in this reaction.

10 Successful combinations are generated by trial and error of locus combinations and by adjustment of primer concentrations to identify an equilibrium in which all included loci may be amplified.

15 Of particular importance in the multiplex system is the size range of amplified alleles produced from the individual loci which will be analyzed together. For ease of analysis with current technologies, systems which can be detected by amplification of fragments smaller than 500 bases were preferably selected.

20 The following multiplex combinations have been developed and are considered ideal combinations for use in the present system:

1. HUMTH01 and HUMCSF1PO;
2. HUMTH01 and HUMCD4;
- 25 3. HUMTH01 and HUMTPOX;
4. HUMF13A01 and HUMFABP;
5. HUMF13A01 and HUMMYOPK (Myotonic);
6. HUMF13A01 and HUMBFXIII (F13B);
7. HUMBFXIII (F13B) and HUMFESFPS;
- 30 8. HUMBFXIII (F13B) and HUMLIPOL;
9. HUMHPRTB and HUMFESFPS;
10. HSAC04 (ACTBP2) and HUMCYP19;
11. HSAC04 (ACTBP2) and HUMFABP;
12. HUMCSF1PO, HUMTPOX and HUMTH01;
- 35 13. HUMHPRTB, HUMFESFPS and HUMVWFA31;

14. HSAC04 (ACTBP2), HUMCYP19 and HUMPLA2A1;  
15. HUMAPOA2, HUMCYP19 and HUMPLA2A1;  
16. HUMCD4, HUMCSF1PO and HUMTH01;  
17. HUMCYP19, HUMFABP and HUMPLA2A1;  
5 18. HUMCYP19, HUMHPRTB and HUMPLA2A1;  
19. HUMF13A01, HUMFABP and HUMCD4;  
20. HUMHPRTB, HUMFESFPS and HUMLIPOL;  
21. HUMF13A01, HUMFABP and HUMCD4;  
22. HUMHPRTB, HUMBFXIII (F13B) and HUMPLA2A1;  
10 23. HUMHPRTB, HUMBFXIII (F13B) and HUMTPOX;  
24. HUMHPRTB, HUMBFXIII (F13B) and HUMFESFPS;  
25. HUMCSF1PO, HUMTPOX and HUMCD4;  
26. HUMHPRTB, HUMFESFPS and HUMMYOPK (Myotonic);  
27. HUMCSF1PO, HUMTH01 and HUMCD4;  
15 28. HUMCSF1PO, HUMTH01 and HUMVWFA31;  
29. HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL;  
30. HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31;  
31. HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B) and  
HUMLIPOL;  
20 32. HUMCSF1PO, HUMTPOX, HUMTH01 and HUMCD4; and  
33. HUMCSF1PO, HUMTH01, HUMTPOX and HUMCD4.

The primers must also be designed so that the size  
of the resulting amplification products differ in length,  
25 thereby facilitating assignment of alleles to individual loci  
during detection. Inappropriate selection of primers can  
produce several undesirable effects such as lack of  
amplification, amplification at multiple sites, primer dimer  
formation, undesirable interaction of primer sequences from  
30 different loci, production of alleles from one locus which  
overlap with alleles from another, or requirement for  
amplification conditions or protocols for the different loci  
which are incompatible in a multiplex. The synthesis of the  
35 primers is conducted by procedures known to those skilled in  
the art.

Using Multiplexes of Two Loci to Develop Multiplexes Using More than Two Loci

Once a multiplex containing two loci is developed,  
5 it may be used as a core to create multiplexes containing more than two loci. New combinations are created including the first two loci. For example, the core multiplex containing loci HUMTH01 and HUMCSF1PO was used to generate derivative multiplexes of HUMTH01, HUMCSF1PO, and HUMTPOX; HUMTH01,  
10 HUMCSF1PO, and HUMCD4; HUMTH01, HUMCSF1PO, and HUMVWFA31; HUMTH01, HUMCSF1PO, HUMVWFA31, and HUMTPOX; and HUMTH01, HUMCSF1PO, HUMCD4, and HUMTPOX. Many other derivative multiplexes can be generated based upon a working multiplex. The derivative multiplexes are, in some sense, routine  
15 extensions of the core multiplex. /

Preparation of Genomic DNA

All methods of DNA preparation which are compatible with the amplification process for a single locus should be appropriate for multiplex amplification. Many examples of preparation methods have been described in the literature  
20 (Patel et al. 1984, Gill et al. 1985). DNA concentrations are measured fluorometrically (Brunk et al. 1979).

Amplification of DNA

Human genomic DNA samples are subjected to PCR  
25 amplification using primers and thermocycling conditions specific for each locus. Reference is made to Table 1 for details of the primer sequences. The amplification protocol specific to each multiplex is listed in the specific examples.

Designation	Primer sequences	Sequence ID Number
5	HSAC04 (ACTBP2) primer 1: ACA TCT CCC CTA CCG CTA TA primer 2: AAT CTG GGC GAC AAG AGT GA	1 2
	HUMAPOA2 (APOCIII) primer 1: GGA GCA GTC CTA GGG CCG CGC CGT primer 2: GTG ACA GAG GGA GAC TCC ATT AAA	3 4
	HUMCSF1PO primer 1: AAC CTG AGT CTG CCA AGG ACT AGC primer 2: TTC CAC ACA CCA CTG GCC ATC TTC	5 6
	HUMCYP19 (CYARP450) primer 1: GCA GGT ACT TAG TTA GCT AC primer 2: TTA CAG TGA GCC AAG GTC GT	7 8
10	HUMCD4 primer 1: CCA GGA AGT TGA GGC TGC AGT GAA primer 2: TTG GAG TCG CAA GCT GAA CTA GCG	9 10
	HUMF13A01 primer 1: GAG GTT GCA CTC CAG CCT TTG CAA primer 2: TTC CTG AAT CAT CCC AGA GCC ACA	11 12
	HUMBFXIII (F13B) primer 1: TGA GGT GGT GTC CTA CCA TA primer 2: GAT CAT GCC ATT GCA CTC TA	13 14
	HUMFABP primer 1: GTA GTA TCA GTT TCA TAG GGT CAC C primer 2: CAG TTC GTT TCC ATT GTC TGT CCG	15 16
15	HUMFESFPS primer 1: GCT GTT AAT TCA TGT AGG GAA GGC primer 2: GTA GTC CCA GCT ACT TGG CTA CTC	17 18
	HUMHPRTB (HPRT-1) primer 1: ATG CCA CAG ATA ATA CAC ATC CCC primer 2: CTC TCC AGA ATA GTT AGA TGT AGG	19 20
	HUMMYOPK Myotonic primer 1: GCT CGA AGG GTC CTT GTA GCC GGG primer 2: GAT AGG TGG GGG TGC GTG GAG GAT	21 22
	HUMLIPOL primer 1: CTG ACC AAG GAT AGT GGG ATA TAG primer 2: GGT AAC TGA GCG AGA CTG TGT CT	23 24
20	HUMPLA2A1 (PLA-AZ) primer 1: GGT TGT AAG CTC CAT GAG GTT AGA primer 2: TTG AGC ACT TAC TAT GTG CCA GGC T	25 26
	HUMTH01 primer 1: GTG GGC TGA AAA GCT CCC GAT TAT primer 2: ATT CAA AGG GTA TCT GGG CTC TGG	27 28
	HUMTPOX primer 1: ACT GGC ACA GAA CAG GCA CTT AGG primer 2: GGA GGA ACT GGG AAC CAC ACA GGT	29 30
	HUMVWFA31 primer 1: GA AAG CCC TAG TGG ATG ATA AGA ATA ATC primer 2: GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG	31 32

Reference is made to the examples below for additional details of the specific procedure relating to each multiplex. The locus-specific primers include a number of nucleotides which, under the conditions used in the hybridization, are sufficient to hybridize with an allele of the locus to be amplified and to be essentially free from amplification of alleles of other loci. Reference is made to U. S. Patent 5,192,659 to Simons, which is incorporated herein by reference for a more detailed description of locus-specific primers.

Separation and Detection of DNA Fragments

Following amplification, products are then separated by electrophoresis, e.g., denaturing polyacrylamide

gel electrophoresis (Sambrook et al., 1989). Preferred gel preparation and electrophoresis procedures are conducted as described in Example 1. Fragment separation occurs based on size and charge of the sample.

5 The DNA is then detected by, e.g., silver staining (Bassam et al. 1991). Alternatively, if radioactively-labeled or fluorescently-labeled primers were used for each locus, the products are detected by means available to detect these reporters as known to those skilled in the art. Amplified 10 materials may be detected using any of a number of reporters including, e.g., silver staining, radioisotopes, fluorescers, chemiluminescers and enzymes in combination with detectable substrates.

15 Individual DNA samples containing amplified alleles are preferably compared with a size standard such as a DNA marker or locus-specific allelic ladder to determine the alleles present at each locus within the sample. The preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci 20 consists of a combination of allelic ladders for the loci being evaluated.

25 The preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci which are generated using fluorescently-labeled primers for each locus consists of a combination of fluorescently-labeled allelic ladders for the loci being evaluated.

30 Following the construction of allelic ladders for individual loci, they may be mixed and loaded for gel electrophoresis at the same time as the loading of amplified samples occurs. Each allelic ladder co-migrates with alleles in the sample from the corresponding locus.

35 A permanent record of the data can be generated with the use of electrophoresis duplicating film (STR systems manual #TMD004, Promega Corporation, Madison, WI.).

Advantage of Fluorescent Detection

With the advent of automated fluorescent imaging, faster detection and analysis of multiplex amplification products can be achieved. For fluorescent analyses, one 5 fluoresceinated primer can be included in the amplification of each locus. Separation of the amplified fragments is achieved in precisely the same manner as with the silver stain detection method. The resulting gel is loaded onto a FluorImager® 575 (Molecular Dynamics, Sunnyvale, CA) which 10 scans the gel and digitizes the data in three minutes. The FluorImager® contains an argon laser emitting 488 nm light which sweeps through the gel using a galvanometer-controlled mirror. The light activates fluorescent molecules in its path and they, in turn, emit light of higher wavelength. A 15 filter prohibits passage of the original light, but allows collection of the emitted light by a fiber optic collector. A second filter selected by the user may be inserted between the fiber optic collector and the photomultiplier, allowing detection of specific wavelength bands (or colors) with each 20 scan.

The image has an overall cleaner appearance than that obtained with the silver stain for three reasons. First, only one of the two PCR product strands is labeled with primer, simplifying the two band per allele images of 25 the silver stain. Second, in the silver stain reaction, the entire gel is exposed to silver and prone to silver deposition causing a significant general background. With the fluorescent reporter, only the primer is labeled and the unincorporated primers migrate out of the bottom of the gel 30 prior to detection. Third, some artifact bands of the PCR reaction are plentiful, but contain very little primer.

Because this fluorescent method detects only products with one particular primer, some of these artifacts which appear in silver stain of multiplex amplifications are 35 not detected. In fact, this characteristic has allowed

development of the more complex quadriplex as shown in Figure 2 in place of the triplex shown in Figure 1.

#### Kit

The present invention is also directed to kits that 5 utilize the process described. A basic kit includes a container having a locus-specific primer pair (or alternately separate containers containing each primer of a primer pair) for each locus. The kit also includes instructions for use.

Other ingredients may include an allelic ladder 10 directed to each of the specified loci, a sufficient quantity of enzyme for amplification, amplification buffer to facilitate the amplification, loading solution for preparation of the amplified material for gel electrophoresis, human genomic DNA as a control to test that 15 the system is working well, a size marker to insure that materials migrate as anticipated in the gel, and a protocol and manual to educate the user and to limit error in use. The amounts of the various reagents in the kits can be varied depending upon a number of factors, such as the optimum 20 sensitivity of the process. The instructions for use are suitable to enable any analyst to carry out the desired test. It is within the scope of this invention to provide test kits for use in manual applications or test kits for use with automated detectors or analyzers.

25

#### EXAMPLES

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. The examples 30 are not intended in any way to otherwise limit the scope of the disclosure or protection granted by the patent.

Genomic DNA isolation and quantitation were performed essentially as described by Puers et al., 1993. These methods are generally known to those skilled in the art 35 and are preferred, but not required, for application of the

invention.

Amplification products were separated by electrophoresis through a 0.4mm thick 4% denaturing polyacrylamide gel (19:1 ratio of acrylamide to bis-acrylamide) which contained 7 M urea (Sambrook et al., 1989) and was chemically cross-linked to one glass plate (Kobayashi, 1988). DNA samples were mixed with 3 $\mu$ l loading solution (10mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C for 2 min., and chilled on ice prior to loading.

Electrophoresis was performed at 60 W in 0.5x TBE for 1-2 hrs. The DNA was detected by silver staining (Bassam et al., 1991). Permanent images were obtained by exposure to Electrophoresis Duplicating Films (EDF, Kodak, Cat. No. 809 6232). Alternatively, detection can be performed by fluorescent scanning (Schumm et al., 1994) or radioactive detection (Hammond et al., 1994).

#### EXAMPLE 1

##### 20 Silver Stain Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, and HUMTH01

In this example, a DNA template (three DNA samples) was amplified at the individual loci HUMCSF1PO, HUMTPOX, and HUMTH01 simultaneously in a single reaction vessel. The PCR amplifications were performed in 50 $\mu$ l volumes using 25ng template, 0.03U Taq DNA Polymerase/ $\mu$ l, 1x STR Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.5mM MgCl<sub>2</sub> and 200 $\mu$ M each of dATP, dCTP, dGTP and dTTP), and using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 1 (96°C for 2 min., then 10 cycles of 94°C for 1 min., 64°C for 1 min., and 70°C for 1.5 min., followed by 20 cycles of 90°C for 1 min., 64°C for 1 min., 70°C for 1.5 min.) was employed.

35 Six amplification primers were used in combination, including 0.2 $\mu$ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2

[SEQ. ID. 6], 0.2 $\mu$ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 0.6 $\mu$ M each HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28].

5 Amplified products were separated by denaturing acrylamide gel electrophoresis on a 40cm gel for 60-90 min. at 60 W and products were visualized by silver stain analysis according the protocol of Bassam et al. (1991).

10 Reference is made to Figure 1 which reveals the silver stain detection of the multiplex amplification. Lanes 2, 3, and 5 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, and HUMTH01. Lanes 1, 4, and 7 contain allelic ladders for the three loci and lane 6 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

15

#### EXAMPLE 2

##### Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31

20 In this example, a DNA template was amplified at the individual loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31 simultaneously in a single reaction vessel. The PCR amplifications were performed in 25 $\mu$ l volumes using 25ng template, 0.04U Taq DNA Polymerase/ $\mu$ l, 1x STR Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.5mM MgCl<sub>2</sub> and 200 $\mu$ M each of dATP, dCTP, dGTP and dTTP), and using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 1, as described in Example 1, was employed. Eight amplification primers were used in combination, including 1 $\mu$ M each HUMCSF1PO primer 2 [SEQ. ID. 5] and fluorescein-labeled primer 1 [SEQ. ID. 5], 0.15 $\mu$ M each HUMTPOX primer 1 [SEQ. ID. 29] and fluorescein-labeled primer 2 [SEQ. ID. 30], 0.2 $\mu$ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27], and 1 $\mu$ M each HUMVWFA31 primer 1 [SEQ. ID. 31] and fluorescein-labeled primer 2 [SEQ. ID. 32].

Amplified products were separated by denaturing acrylamide gel electrophoresis on a 32cm gel for 45 minutes at 40 watts. Detection of the fluorescent signal was achieved using the FluorImager® 575 (Molecular Dynamics, Sunnyvale, CA). Reference is made to Figure 2 which is a computer image of a FluorImager scan. Lanes 2-7 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31. Lane 1 contains allelic ladders for the 4 loci.

10

### EXAMPLE 3

#### Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMVWFA31

15 In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMVWFA31 simultaneously in a single reaction vessel. The PCR amplifications were performed in 25 $\mu$ l volumes using 25ng template, 0.03U Taq DNA Polymerase/ $\mu$ l, 1x STR Buffer (described in example 1), and a 20 Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 2 (96°C for 2 min., then 10 cycles of 94°C for 1 min., 60°C for 1 min., and 70°C for 1.5 min., followed by 20 cycles of 90°C for 1 min., 64°C for 1 min., 70°C for 1.5 min.) was employed. Amplified products were separated by 25 denaturing acrylamide gel electrophoresis on a 32cm gel for 45 min. at 40 W and products were visualized by silver stain analysis according the protocol of Bassam et al. (supra.). Six primers were used in combination including 0.2 $\mu$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 1.5 $\mu$ M 30 each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 1 $\mu$ M each HUMVWFA31 primers 1 [SEQ. ID. 31] and 2 [SEQ. ID. 32].

35 Reference is made to Figure 3 which reveals the silver stain detection of the multiplex amplification. Lanes 2-6 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, and HUMVWFA31. Lanes 1 and 7 contain allelic ladders for the 3 loci..

## EXAMPLE 4

Fluorescent Detection of Multiplex Amplification of Loci  
HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and HUMLIPOL

5 In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 2 using amplification protocol 2, as  
10 described in Example 3.

Eight primers were used in combination, including 1 $\mu$ M each HUMHPRTB primer 2 [SEQ. ID. 20] and fluorescein-labeled primer 1 [SEQ. ID. 19], 2.5 $\mu$ M each HUMFESFPS primer 2 [SEQ. ID. 18] and fluorescein-labeled primer 1 [SEQ. ID. 17],  
15 1 $\mu$ M each HUMBFXIII (F13B) primer 2 [SEQ. ID. 14] and fluorescein-labeled primer 1 [SEQ. ID. 13], and 0.5 $\mu$ M each HUMLIPOL primer 2 [SEQ. ID. 24] and fluorescein-labeled primer 1 [SEQ. ID. 23].

Reference is made to Figure 4 which is a computer  
20 image of a FluorImager scan. Lanes 2-7 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and HUMLIPOL. Lane 1 contains allelic ladders for the 4 loci.

25

## EXAMPLE 5

Multiplex Amplification of Loci HSAC04  
(ACTBP2) and HUMCYP19

In this example, a DNA template was amplified at  
30 the individual loci HSAC04 and HUMCYP19 simultaneously in a single reaction vessel. The PCR amplifications were performed in 15 $\mu$ l volumes with 25ng template, 0.01U Taq DNA Polymerase/ $\mu$ l, 1x Taq DNA Polymerase Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100 and 1.5mM MgCl<sub>2</sub>)  
35 and 200 $\mu$ M each of dATP, dCTP, dGTP and dTTP using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 2, as described in Example 3, was employed. Amplified products

were separated and detected per example 1. Four primers were used in combination, including 1 $\mu$ M each HSAC04 (ACTBP2) primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], and 1 $\mu$ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8].

5 Reference is made to Figure 5 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HSAC04 (ACTBP2) and HUMCYP19. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a  
10 negative control.

#### EXAMPLE 6

##### Multiplex Amplification of Loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1

15 In this example, a DNA template was amplified at the loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications were performed in 15 $\mu$ l volumes with 25ng  
20 template, 0.02U Taq DNA Polymerase/ $\mu$ l, 1x Taq DNA Polymerase Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100 and 1.5mM MgCl<sub>2</sub>) and 200 $\mu$ M each of dATP, dCTP, dGTP and dTTP using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 2, as described in Example 3, was  
25 employed. Amplified products were separated and detected per example 1. Six primers were used in combination, including 1 $\mu$ M each HSAC04 (ACTBP2) primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], 1 $\mu$ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26], and 1 $\mu$ M each HUMCYP19 primers 1 [SEQ. ID. 7]  
30 and 2 [SEQ. ID. 8].

Reference is made to Figure 6 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

**EXAMPLE 7**Multiplex Amplification of Loci HSAC04  
(ACTBP2) and HUMFABP

5 In this example, a DNA template was amplified at the loci HSAC04 (ACTBP2) and HUMFABP simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 5 using amplification protocol 2, as described in Example 3. Four  
10 primers were used in combination, 1 $\mu$ M each HSAC04 (ACTBP2) primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], and 1 $\mu$ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16].

15 Reference is made to Figure 7 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HSAC04 (ACTBP2) and HUMFABP. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

20

**EXAMPLE 8**Multiplex Amplification of Loci  
HUMAPOA2, HUMCYP19, and HUMPLA2A1

25 In this example, a DNA template was amplified at the loci HUMAPOA2, HUMCYP19, and HUMPLA2A simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 $\mu$ M each HUMAPOA2 primers 1 [SEQ. ID. 3] and 2 [SEQ. ID. 4], 1 $\mu$ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], and 1 $\mu$ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

30 Reference is made to Figure 8 which reveals the silver stain detection of the multiplex amplification. Lanes 1 and 3 contain DNA samples simultaneously co-amplified for the loci HUMAPOA2, HUMCYP19, and HUMPLA2A1. Lane 2 contains a DNA sample which failed to amplify and lane 4 displays a sample

without DNA subjected to the same procedures, i.e., a negative control.

#### EXAMPLE 9

5                   Multiplex Amplification of Loci  
                  HUMCD4, HUMCSF1PO, and HUMTH01

In this example, a DNA template was amplified at the loci HUMCD4, HUMCSF1PO, and HUMTH01 simultaneously in a 10 single reaction vessel. The PCR amplifications were performed in 50 $\mu$ l volumes with 25ng template, 0.02U Taq DNA Polymerase/ $\mu$ l, 1x Taq DNA Polymerase Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100 and 1.5mM MgCl<sub>2</sub>) and 200 $\mu$ M each of dATP, dCTP, dGTP and dTTP using a Thermal 15 Cycler 480 (Perkin Elmer Cetus). Amplification protocol 1, as described in Example 1, was employed. Amplified products were separated and detected as described in Example 1. Six 20 primers were used in combination, including 1 $\mu$ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10], 1 $\mu$ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], and 1 $\mu$ M each HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28].

Reference is made to Figure 9 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the 25 loci HUMCD4, HUMCSF1PO, and HUMTH01. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

#### EXAMPLE 10

30                   Multiplex Amplification of Loci  
                  HUMCYP19, HUMFABP, and HUMPLA2A1

In this example, a DNA template was amplified at the loci HUMCYP19, HUMFABP, and HUMPLA2A1 simultaneously in 35 a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 2, as described in Example 3. Six

primers were used in combination, including 1 $\mu$ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], 1 $\mu$ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16] and 1 $\mu$ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

5 Reference is made to Figure 10 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCYP19, HUMFABP, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a 10 negative control.

#### EXAMPLE 11

##### Multiplex Amplification of Loci HUMCYP19, HUMHPRTB, and HUMPLA2A1

15 In this example, a DNA template was amplified at the loci HUMCYP19, HUMHPRTB, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 9 using 20 amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 $\mu$ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], 1 $\mu$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], and 1 $\mu$ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

25 Reference is made to Figure 11 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCYP19, HUMHPRTB, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a 30 negative control.

#### EXAMPLE 12

##### Multiplex Amplification of Loci HUMF13A01 and HUMFABP

35 In this example, a DNA template was amplified at the loci HUMF13A01 and HUMFABP simultaneously in a single reaction vessel. The PCR amplifications and other

manipulations were performed as described in Example 5 using amplification protocol 1, as described in Example 1. Four primers were used in combination, including 1 $\mu$ M each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12], and 5 1 $\mu$ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16].

Reference is made to Figure 12 which reveals the silver stain detection of the multiplex amplification. Lanes 10 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01 and HUMFABP. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative 15 control.

#### EXAMPLE 13

##### Multiplex Amplification of Loci HUMBFXIII (F13B) and HUMFESFPS

In this example, a DNA template was amplified at the loci HUMBFXIII (F13B) and HUMFESFPS simultaneously in a single reaction vessel. The PCR amplifications and other 20 manipulations were performed as described in Example 6 using amplification protocol 1, as described in Example 1. Four primers were used in combination, including 1 $\mu$ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14], and 1 $\mu$ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. 25 ID. 18].

Reference is made to Figure 13 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B) and HUMFESFPS. Lane 4 displays a 30 sample without DNA subjected to the same procedures, i.e., a negative control.

**EXAMPLE 14**Multiplex Amplification of Loci  
HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1

5       In this example, a DNA template was amplified at the loci HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 2, as 10 described in Example 3. Six primers were used in combination, including 1 $\mu$ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14], 1 $\mu$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], and 1 $\mu$ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

15       Reference is made to Figure 14 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same 20 procedures, i.e., a negative control.

**EXAMPLE 15**Multiplex Amplification of Loci  
HUMF13A01, HUMFABP, and HUMCD4

25       In this example, a DNA template was amplified at the loci HUMF13A01, HUMFABP, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 5 using 30 amplification protocol 1, as described in Example 1. Six primers were used in combination, including 1 $\mu$ M each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12], 1 $\mu$ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16], and 1 $\mu$ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

35       Reference is made to Figure 15 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the

loci HUMF13A01, HUMFABP, and HUMCD4. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

5

#### EXAMPLE 16

##### Multiplex Amplification of Loci HUMHPRTB and HUMFESFPS

In this example, a DNA template was amplified at 10 the loci HUMHPRTB and HUMFESFPS simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using 500-0.5ng template, 0.02U Taq DNA Polymerase/ $\mu$ l and amplification protocol 2, as described in Example 3. Four 15 primers were used in combination, including 0.2 $\mu$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20] and 1.5 $\mu$ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18].

Reference is made to Figure 16 which reveals the 20 silver stain detection of the multiplex amplification. Lanes 1-6 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB and HUMFESFPS using 500, 50, 25, 5, 1 and 0.5ng DNA template. Lane 7 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

25

#### EXAMPLE 17

##### Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMLIPOL

In this example, a DNA template was amplified at 30 the loci HUMHPRTB, HUMFESFPS, and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six 35 primers were used in combination, including 0.4 $\mu$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 3 $\mu$ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18],

and 2 $\mu$ M each HUMLIPOL primers 1 [SEQ. ID. 23] and 2 [SEQ. ID. 24].

Reference is made to Figure 17 which reveals the silver stain detection of the multiplex amplification. Lanes 5 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS and HUMLIPOL. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

10

#### EXAMPLE 18

##### Multiplex Amplification of Loci HUMBFXIII (F13B) and HUMLIPOL

In this example, a DNA template was amplified at 15 the loci HUMBFXIII (F13B) and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using 0.02U Taq DNA Polymerase/ $\mu$ l and amplification protocol 2, as described in Example 3. Four primers were used in 20 combination, including 1 $\mu$ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14] and 1 $\mu$ M each HUMLIPOL primers 1 [SEQ. ID. 23] and 2 [SEQ. ID. 24].

Reference is made to Figure 18 which reveals the silver stain detection of the multiplex amplification. Lanes 25 1-3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B) and HUMLIPOL.

30

#### EXAMPLE 19

##### Multiplex Amplification of Loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B)

In this example, a DNA template was amplified at 35 the loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six primers were used in

combination, including 1 $\mu$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 0.2 $\mu$ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 2 $\mu$ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

5 Reference is made to Figure 19 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B).

10

#### EXAMPLE 20

##### Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMBFXIII (F13B)

In this example, a DNA template was amplified at 15 the loci HUMHPRTB, HUMFESFPS, and / HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six primers were used in 20 combination, including 1 $\mu$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 2 $\mu$ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 2 $\mu$ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

Reference is made to Figure 20 which reveals the 25 silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, and HUMBFXIII (F13B).

30

#### EXAMPLE 21

##### Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, and HUMCD4

In this example, a DNA template was amplified at 35 the loci HUMCSF1PO, HUMTPOX, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 1, as described in Example 1. Six

primers were used in combination, including 1 $\mu$ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], 1 $\mu$ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 1 $\mu$ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

5 Reference is made to Figure 21 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, and HUMCD4.

10

#### EXAMPLE 22

##### Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMMYOPK (Myotonic)

In this example, a DNA template was amplified at 15 the loci HUMHPRTB, HUMFESFPS, and HUMMYOPK simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six 20 primers were used in combination, including 1 $\mu$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 1 $\mu$ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 1 $\mu$ M each HUMMYOPK (Myotonic) primers 1 [SEQ. ID. 21] and 2 [SEQ. ID. 22].

Reference is made to Figure 22 which reveals the 25 silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, and HUMMYOPK (Myotonic).

30

#### Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4

In this example, a DNA template was amplified at 35 the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using 0.04U Taq DNA Polymerase/ $\mu$ l and

amplification protocol 1, as described in Example 1. Eight primers were used in combination, including 1 $\mu$ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], 1 $\mu$ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], 1 $\mu$ M each 5 HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28], and 1 $\mu$ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

Reference is made to Figure 23 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified 10 for the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4.

#### EXAMPLE 24

##### Multiplex Amplification of Loci HUMF13A01 and HUMMYOPK (Myotonic)

15 In this example, a DNA template was amplified at the loci HUMF13A01 and HUMMYOPK (Myotonic) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using 20 0.04U Taq DNA Polymerase/ $\mu$ l and amplification protocol 1, as described in Example 1. Four primers were used in combination, including 0.1 $\mu$ M each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12] and 1 $\mu$ M each HUMMYOPK (Myotonic) primers 1 [SEQ. ID. 21] and 2 [SEQ. ID. 22].

25 Reference is made to Figure 24 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01 and HUMMYOPK (Myotonic).

30

#### EXAMPLE 25

##### Multiplex Amplification of Loci HUMF13A01 and HUMBFXIII (F13B)

35 In this example, a DNA template was amplified at the loci HUMF13A01 and HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using

0.03U Taq DNA Polymerase/ $\mu$ l and amplification protocol 2, as described in Example 3. Four primers were used in combination, including 0.1 $\mu$ M each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12] and 0.5 $\mu$ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

5 Reference is made to Figure 25 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01 and HUMBFXIII (F13B).

10

#### EXAMPLE 26

##### Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4

15 In this example, a DNA template was amplified at the individual loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.04U Taq DNA Polymerase/ $\mu$ l and amplification protocol 1, as described in Example 1. Eight amplification primers were used in combination, including 2 $\mu$ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 0.5 $\mu$ M each HUMTPOX primer 1 [SEQ. ID. 29] and fluorescein-labeled primer 2 [SEQ. ID. 30], 0.5 $\mu$ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 0.5 $\mu$ M each HUMCD4 primer 1 [SEQ. ID. 9] and fluorescein-labeled primer 2 [SEQ. ID. 10].

20 25 30 Amplified products were detected as in Example 2. Reference is made to Figure 26 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4.

## EXAMPLE 27

Fluorescent Detection of Multiplex Amplification  
of Loci HUMCSF1PO, HUMTH01, and HUMCD4

5 In this example, a DNA template was amplified at the individual loci HUMCSF1PO, HUMTH01, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02U Taq DNA Polymerase/ $\mu$ l and amplification protocol 1, as 10 described in Example 1. Six amplification primers were used in combination, including 1 $\mu$ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 1 $\mu$ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 $\mu$ M each HUMCD4 primer 1 [SEQ. ID. 9] and fluorescein-labeled primer 2 [SEQ. ID. 10]. 15

Amplified products were detected as in Example 2. Reference is made to Figure 27 which is a photograph of a computer image of a FluorImager scan. Lanes 1 and 2 contain DNA samples simultaneously co-amplified for the loci 20 HUMCSF1PO, HUMTH01, and HUMCD4.

## EXAMPLE 28

Fluorescent Detection of Multiplex Amplification  
of Loci HUMCSF1PO, HUMTH01, and HUMVWFA31

25 In this example, a DNA template was amplified at the individual loci HUMCSF1PO, HUMTH01, and HUMVWFA31 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 30 0.02U Taq DNA Polymerase/ $\mu$ l and amplification protocol 1, as described in Example 1. Six amplification primers were used in combination, including 1 $\mu$ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 1 $\mu$ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27], and 1 $\mu$ M each HUMVWFA31 primer 1 [SEQ. ID. 31] and fluorescein-labeled primer 2 [SEQ. ID. 32]. 35

Amplified products were detected as in Example 2.

Reference is made to Figure 28 which is a photograph of a computer image of a FluorImager scan. Lanes 1 and 2 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTH01, and HUMVWFA31.

5

#### EXAMPLE 29

##### Fluorescent Detection of Multiplex Amplification of Loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL

10 In this example, a DNA template was amplified at the individual loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.03U Taq DNA Polymerase/ $\mu$ l and amplification protocol 2, as 15 described in Example 3. Six amplification primers were used in combination, including 1 $\mu$ M each HUMHPRTB primer 2 [SEQ. ID. 20] and fluorescein-labeled primer 1 [SEQ. ID. 19], 1 $\mu$ M each HUMBFXIII (F13B) primer 2 [SEQ. ID. 14] and fluorescein-labeled primer 1 [SEQ. ID. 13], and 1 $\mu$ M each HUMLIPOL primer 20 2 [SEQ. ID. 24] and fluorescein-labeled primer 1 [SEQ. ID. 23].

25 Amplified products were detected as in Example 2. Reference is made to Figure 29 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL.

#### EXAMPLE 30

##### Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO and HUMTH01

30 In this example, a DNA template was amplified at the individual loci HUMCSF1PO and HUMTH01 simultaneously in a single reaction vessel. The PCR amplifications were 35 performed as described in Example 1 using 0.02U Taq DNA Polymerase/ $\mu$ l and amplification protocol 1, as described in Example 1. Four amplification primers were used in

combination, including 2 $\mu$ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5] and 1 $\mu$ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27].

5           Amplified products were detected as in Example 2. Reference is made to Figure 30 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO and HUMTH01.

10

#### EXAMPLE 31

##### Fluorescent Detection of Multiplex Amplification of Loci HUMTH01 and HUMCD4

15           In this example, a DNA template was amplified at the individual loci HUMTH01 and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02U Taq DNA Polymerase/ $\mu$ l and amplification protocol 1, as described in Example 1.

20           Four amplification primers were used in combination, including 1 $\mu$ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 $\mu$ M each HUMCD4 primer 1 [SEQ. ID. 9] and fluorescein-labeled primer 2 [SEQ. ID. 10].

25           Amplified products were detected as in Example 2. Reference is made to Figure 31 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMTH01 and HUMCD4.

30

#### EXAMPLE 32

##### Fluorescent Detection of Multiplex Amplification of Loci HUMTH01 and HUMTPOX

35           In this example, a DNA template was amplified at the individual loci HUMTH01 and HUMTPOX simultaneously in a single reaction vessel. The PCR amplifications were performed

as described in Example 1 using 0.02U Taq DNA Polymerase/ $\mu$ l and amplification protocol 1, as described in Example 1. Four amplification primers were used in combination, including 1 $\mu$ M each HUMTH01 primer 2 [SEQ. ID. 28] and 5 fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 $\mu$ M each HUMTPOX primer 2 [SEQ. ID. 30] and fluorescein-labeled primer 1 [SEQ. ID. 29].

Amplified products were detected as in Example 2. Reference is made to Figure 32 which is a photograph of a 10 computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMTH01 and HUMTPOX.

It is understood that the invention is not confined to the particular construction and arrangements herein 15 illustrated and described, but embraces such modified forms thereof and come within the scope of the claims following the bibliography.

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